Biochem. J. (1960) 75, 483

Trace Elements and the Synthesis of Vitamin B_{12} by *Streptomyces olivaceus*

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(*Received* 15 *October* 1959)

Study of the requirement of trace elements for the streptomycetes has often led to contradictory results (Woodruff, 1947; Spilsbury, 1948; Johnstone & Waksman, 1948). This is mostly due to the difficulty in preparing a truly basal medium and also because such requirements vary with the medium employed (Thornberry & Anderson, 1948). The streptomycete *Streptomyces olivaceus* is one of the few micro-organisms producing significant amounts of vitamin B_{12} (Hall, Benedict, Wiesen, Smith & Jackson, 1953; Hester & Ward, 1954; Pfeifer, Vojnovich & Heger, 1954). It was therefore thought pertinent to make a comparative study of the effect of supplementation of a synthetic basal medium for *S. olivaceus* with various trace elements, singly or in combination, on the growth of mycelia and the synthesis of the vitamin.

EXPERIMENTAL

Organism. The organism used throughout was *Streptomyces olivaceus* NRRL B-1125, kindly provided by Dr C. W. Hesseltine of the Northern Utilization Research and Development Division, Peoria, U.S.A. Details about its maintenance have been reported earlier (Maitra & Roy, 1959*a*).

Media. The medium described by Dulaney & Williams (1953) was modified slightly and contained (per l.): glucose, 20·0 g.; $(NH_4)_2HPO_4$, 7·5 g.; NaCl, 5·0 g.; K_2HPO_4, 1·0 g.; KH_2PO_4, 1·0 g.; $MgSO_4,7H_2O$, 1·0 g.; $CaCO_3$, 1·0 g.; $CaCl_2$, 0·4 g.; $FeSO_4,7H_2O$, 10·0 mg.; $ZnSO_4,7H_2O$, 10·0 mg.; $CoSO_4,7H_2O$, 10·0 mg.

Removal of trace elements. The water used was deionized and distilled twice from an all-glass still. The conductivity determined after boiling for 5 min. was $0·72 \times 10^{-6}$ mho at 34° as measured with a Leeds–Northrup conductivity bridge. All the glassware used was either of Pyrex or Jena brand. It was washed first with a detergent, rinsed thoroughly in tap water, soaked for 24 hr. in chromic acid, then washed at least 50 times in tap water, rinsed 10 times with glass-distilled water and finally five times with the deionized double-distilled water and drained dry. Glucose was purified by passing a 20% soln. twice through a column of scrupulously washed Amberlite 1R-120(H^+). The other chemicals used were 'Guaranteed Reagents' from E. Merck and Co. Calcium carbonate was washed with stirring and subsequent settling with 20 changes of deionized double-distilled water. The paste was finally dried in a vacuum desiccator. The sulphates of zinc, cobalt, nickel, manganese, magnesium, chromium and copper were recrystallized once from deionized double-distilled water. The water of crystallization in the recrystallized sample of $Cr_2(SO_4)_3$ was determined, by analysis of a standard solution of the dried sample for H_2SO_4 liberated by passing a portion of it through Amberlite 1R-120(H^+), and was found to be 18. The other salts were used without further purification. To minimize heavy-metal contamination $(NH_4)_2HPO_4$, K_2HPO_4, KH_2PO_4 and NaCl were treated with $CaCO_3$ before use according to Steinberg (1950). In preparing media the element under test was first omitted from and then added to the basal medium in graded doses. Calcium carbonate was added separately to each flask, as was glucose after separate sterilization.

Culture conditions. Except where otherwise stated the inoculum was grown in 100 ml. of the medium described before with no added $CaCO_3$ in 500 ml. Erlenmeyer flasks. The fermentation experiments were conducted in 25 ml. of medium in 100 ml. Erlenmeyer flasks. Incubations were carried out on a rotary shaker (120 rev./min.) at room temperature (about 30°), the periods of inoculum preparation being 96 hr. and that of fermentation proper being 120 hr. Inoculation into the inoculum medium was done with a platinum wire with a pin-point of the stock culture and, after the required period, the suspension was centrifuged at 1500 *g* at 0° to separate cells from the liquid broth, a further 10 washes being made with 150-ml. portions of sterile 0·85% KCl. A weighed portion of the sedimented mycelia was suspended in 0·85% KCl and 0·1 ml. of this suspension (equivalent approximately to 1–2 μg. of N) was used to inoculate each flask containing 25 ml. of the medium.

Analytical procedures. The amounts of mycelia and the vitamin B_{12} produced were determined by methods described by Maitra & Roy (1959*b*). The fermented broth was heated on a water bath for 15 min. at pH 5·5 and the liberated vitamin was estimated in the supernatant with *Escherichia coli* 113-3, by the filter-paper-disk assay. Duplicate flasks were used for each concentration of the element under test. The ash of *S. olivaceus* was obtained by incinerating in a porcelain crucible to 600° a sample of scrupulously washed cells used for the above-mentioned inoculations.

RESULTS

Effect of iron, zinc and cobalt on growth and synthesis of vitamin B_{12} by Streptomyces olivaceus. Since the medium used by Dulaney & Williams (1953) contained iron, zinc and cobalt ions each at approximately 2 p.p.m., the experiments with these ions were designed to vary the doses of the element under test and to keep those of the other

two fixed at 2 p.p.m. The results shown in Fig. 1 indicate that iron and zinc are both essential for synthesis of vitamin B_{12} but added cobalt, on the contrary, is apparently not required; this was observed in a number of similar experiments. This is presumably due to traces of cobalt being present in some component(s) of the medium, for the addition of even 0·2 p.p.m. of the ion considerably stimulated the process.

Effect of copper, manganese, chromium, molybdenum, nickel and boron on growth and synthesis of vitamin B_{12} by Streptomyces olivaceus. The basal medium to which additions of the test elements were made was as used before, except that the concentrations of iron, zinc and cobalt were lowered respectively to 1·0, 1·0 and 0·2 p.p.m. in both the inoculation and the fermentation media. The results in Figs. 2 and 3 indicate that copper is the most effective element for promotion of growth and of vitamin synthesis whereas nickel is inhibitory to synthesis of vitamin B_{12} at all doses studied.

Interchangeability of magnesium and manganese. Magnesium (100 p.p.m.) is present in the synthetic medium of Dulaney & Williams (1953), whereas manganese is not added. In the present experiment the sum total of concentrations of these two ions was kept at this level, except, of course, where both of them were omitted; in that case, however, the sulphur deficiency was made good by adding Na_2SO_4 to the medium.

The effect of replacing magnesium by manganese is shown in Table 1. The results indicate that although these two metals are mutually replaceable, one or other is necessary for synthesis of vitamin B_{12}; an absolute requirement, however, could not be demonstrated. Furthermore, manganese appears to be more effective than magnesium for both growth and synthesis of the vitamin in *S. olivaceus*.

Effect of adding trace elements to deficient medium. The effect of ions added alone or in combination on growth and synthesis of vitamin B_{12} in *S. olivaceus* was examined in a medium deficient in trace elements. The fermentations were carried out in the modified medium of Dulaney & Williams (1953), from which iron, zinc and cobalt were omitted. The inoculum, however, was grown as before on a medium containing 1·0, 1·0 and 0·2 p.p.m. of iron, zinc and cobalt respectively. One of the sets of flasks contained ash materials from the mycelia of *S. olivaceus* (comprising 7·84% of the dry weight) in place of added trace elements. As the growth rate was very slow, fermentations for this set of experiments were conducted for 180 hr. None of the elements studied when added alone brought about any substantial increase of cell synthesis over that in the unsupplemented medium. Of the various combinations, iron–zinc–cobalt gave the greatest growth and synthesis of vitamin B_{12}; iron–zinc was less effective (Table 2).

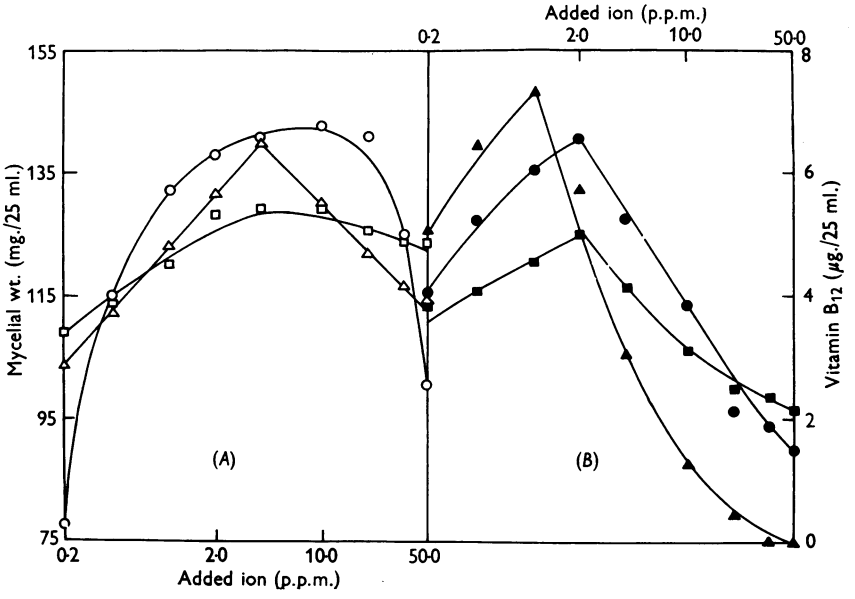

Fig. 1. Effect of iron, zinc and cobalt on mycelial weight and synthesis of vitamin B_{12} in *S. olivaceus*. Representations on the abscissa are in the logarithmic scale. (*A*) Mycelial weight: ○, iron; △, zinc; □, cobalt. The zero levels without addition of ions were 41·8, 57·9 and 95·0 mg./25 ml. respectively. (*B*) Vitamin B_{12} synthesized: ●, iron; ▲, zinc; ■, cobalt. The zero levels without addition of ions were 0, 0 and 1·25 μg./25 ml. respectively.

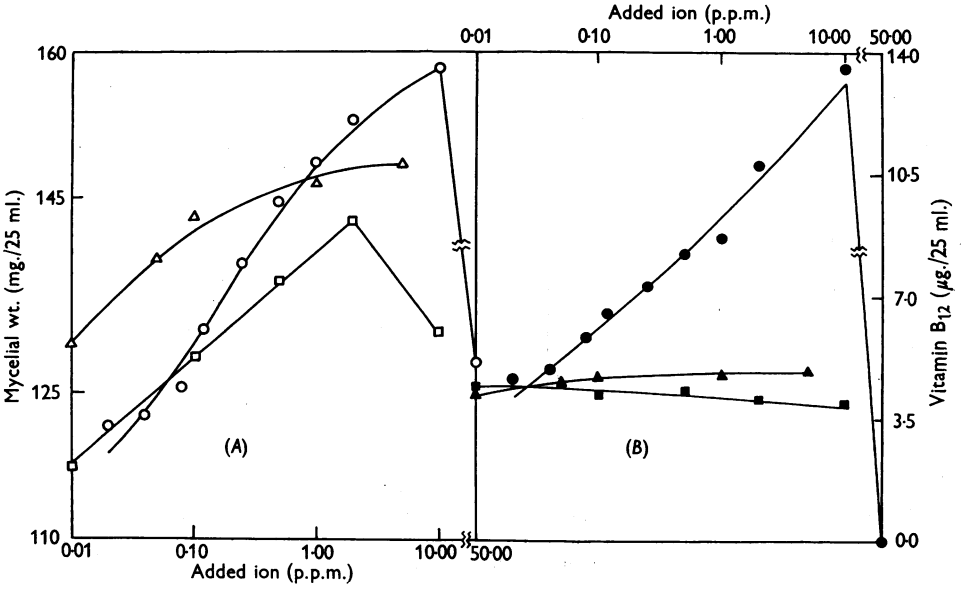

Fig. 2. Effect of copper, manganese and chromium on mycelial weight and synthesis of vitamin B_{12} in *S. olivaceus*. Representations on the abscissa are in the logarithmic scale. (*A*) Mycelial weight: ○, copper; △, manganese; □, chromium. The zero levels without addition of ions were 112·3, 117·4 and 115·9 mg./25 ml. respectively. (*B*) Vitamin B_{12} synthesized: ●, copper; ▲, manganese; ■, chromium. The zero levels without addition of ions were 4·05, 4·15 and 4·20 μg./25 ml. respectively.

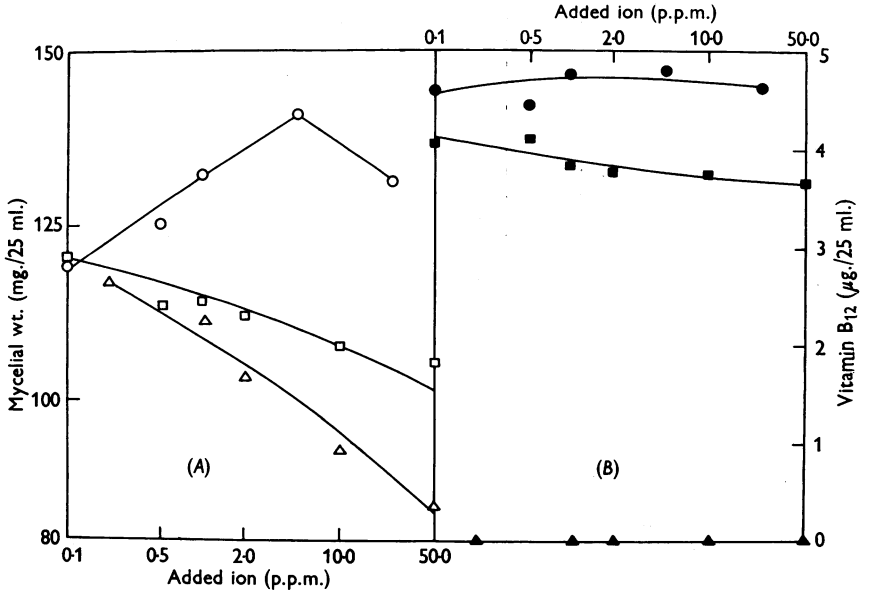

Fig. 3. Effect of molybdenum, nickel and boron on mycelial weight and synthesis of vitamin B_{12} in *S. olivaceus*. Representations on the abscissa are in the logarithmic scale. (*A*) Mycelial weight: ○, molybdenum; △, nickel; □, boron. The zero levels without addition of ions were 117·4, 112·3 and 115·9 mg./25 ml. respectively. (*B*) Vitamin B_{12} synthesized: ●, molybdenum; ▲, nickel; ■, boron. The zero levels without addition of ions were 4·15, 4·05 and 4·20 μg./25 ml. respectively.

Table 1. *Interchangeability of magnesium and manganese in the medium for growth and synthesis of vitamin B_{12} by* Streptomyces olivaceus

Results refer to values for 25 ml. of the medium (details are as given in the text).

Concn. of metals (p.p.m.)		Dry wt. of mycelia	Vitamin B_{12}
Mg	Mn	(mg.)	(μg.)
0	0	90·5	2·25
100	0	118·3	4·25
75	25	146·5	4·90
50	50	144·0	4·75
25	75	149·7	4·80
0	100	149·0	5·00

Table 2. *Effect of trace elements on growth and synthesis of vitamin B_{12} by* Streptomyces olivaceus

Results refer to values for 25 ml. of the medium (details are as given in the text). Incubation was for 180 hr. Figures in parentheses indicate concentration in p.p.m. 'All' indicates the following mixture: Fe (1·0), Zn (1·0), Co (0·2), Cu (1·0), Mo (0·5), Mn (0·1), B (0·1), Cr (0·1) and Ni (0·2).

Additions	Dry wt. of mycelia (mg.)	Vitamin B_{12} (μg.)
None	47·6	0
Fe (1·0)	52·3	Trace
Zn (1·0)	55·6	Trace
Co (0·2)	49·3	0
Cu (1·0)	50·2	0
Mo (0·5)	48·5	0
Mn (0·1)	47·4	0
B (0·1)	43·4	0
Cr (0·1)	46·1	0
Ni (0·2)	36·2	0
Fe (1·0), Zn (1·0)	84·6	1·05
Fe (1·0), Co (0·2)	53·9	0
Zn (1·0), Co (0·2)	45·6	0
Zn (1·0), Ni (0·2)	42·6	0
Fe (1·0), Zn (1·0), Co (0·2)	120·0	3·85
Ni (0·2), Zn (1·0), Co (0·2)	40·8	0
'All'	136·7	0
Ash of *S. olivaceus* mycelium (5·0)	56·2	1·00

DISCUSSION

Heim & Lechevalier (1956) have studied the effect of certain selected ions on the growth of several strains of streptomycetes. Hall *et al.* (1953) studied the requirement of *S. olivaceus* for copper and cobalt in synthesis of vitamin B_{12} in a complex medium and consequently the interpretation of their results is difficult. In the present investigation a synthetic medium has been used to study both growth and synthesis of vitamin B_{12} by *S. olivaceus*. Iron and zinc appear to be necessary for both the processes. Goodwin & McEvoy (1959) have shown that the concentration of iron is critical for flavinogenesis by *Candida flareri*, synthesis being sharply depressed by increasing concentrations of the element. Of a number of trace elements concerned in flavinogenesis, zinc may also be important (Schopfer & Knüsel, 1956). Both in flavinogenesis and synthesis of vitamin B_{12} at least one feature is common, i.e. the formation of nitrogenous ring compounds, and both iron and zinc appear to be involved in the process.

Copper promotes both growth and vitamin B_{12} synthesis in the synthetic medium over a wide range of concentration. Iodice, Richert & Schulman (1958) consider copper to be a component of δ-aminolaevulic acid dehydrase, an enzyme that might possibly be involved in the biosynthesis of the porphyrin-like moiety of vitamin B_{12} (Corcoran & Shemin, 1957). Boron is detrimental to growth, although it does not much inhibit the synthesis of vitamin B_{12}. Such disproportionality between vitamin B_{12} and cell syntheses was reported also by Kurz & Nielsen (1957) working with *Streptomyces griseus*. Chromium, molybdenum and manganese accelerate the growth a little but are practically without effect on synthesis of the vitamin. The inhibitory effect of nickel on both the processes is also significant. The effect of nickel on the decreased synthesis of vitamin B_{12} by *S. olivaceus* was not an artifact of suppressed growth of the assay organism *E. coli* 113–3, since the latter could grow equally well at a concentration of nickel that could inhibit the synthesis of vitamin B_{12} by *S. olivaceus*.

Whether the effect of these trace elements on growth and synthesis of vitamin B_{12} by *S. olivaceus* is direct or indirect is also an important point. Some of the trace elements studied here are either activators or constituents of many enzyme systems (Neilands & Stumpf, 1958) and may be concerned in the growth and synthesis of vitamin B_{12} by *S. olivaceus* in this way.

SUMMARY

1. A glucose–inorganic salt medium has been rendered as free as possible from extraneous metal ions, and the effect of some trace elements on growth and synthesis of vitamin B_{12} by *Streptomyces olivaceus* has been studied.

2. In such a system iron and zinc are essential for synthesis of vitamin B_{12} and cobalt at low levels is stimulatory.

3. Copper favours both growth and synthesis of vitamin B_{12} up to 10 p.p.m. Molybdenum, chromium and manganese are favourable for growth up to 5, 2 and 100 p.p.m. respectively but have no significant effect on the synthesis of the vitamin.

4. Nickel is inhibitory to both growth and synthesis of the vitamin at concentrations as low as 0·2 p.p.m., whereas boron inhibits only the growth.

5. Magnesium can be totally replaced by manganese in the medium.

We are grateful to the Council of Scientific and Industrial Research, New Delhi, for sponsoring the project and for a Fellowship to one of us (P.K.M.), and to Professor B. C. Guha for his kind interest in the work. The skilled technical assistance of Mr D. K. Bose is appreciated. We are also grateful to Dr N. C. Ganguli for useful criticism.

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The Effect of Insulin *in vitro* on the Accumulation of Amino Acids by Isolated Rat Diaphragm

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(*Received* 12 *October* 1959)

It is now well established that insulin *in vitro* enhances the incorporation of labelled amino acids into the protein of isolated rat diaphragm (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958*a*; Wool & Krahl, 1959*a*). This effect of insulin, which is not reproduced *in vitro* by any other hormones examined in this respect (Manchester & Young, 1959; Manchester & Young, in preparation), is not dependent upon or affected by the presence of glucose in the medium, and addition of glucose alone under most conditions has no observable effect on amino acid incorporation.

Since insulin accelerates the rate of entry of glucose and of a variety of non-utilizable sugars into the muscle cell (Levine & Goldstein, 1955; Park, Bornstein & Post, 1955; Helmreich & Cori, 1957; Park, Johnson, Wright & Batsel, 1957), it seemed to us possible that the stimulation by insulin of incorporation of labelled amino acids into the protein of diaphragm might be a consequence of an enhancement by this hormone of the rate of entry of amino acids into the tissue cells. Evidence in support of such a view was found by Kipnis & Noall (1958), when they showed that insulin *in vitro* enhanced the rate of accumulation, and the maximum accumulation effected, of a non-utilizable amino acid (α-amino*iso*butyric acid) by the isolated intact diaphragm preparation of Kipnis & Cori (1957). We have reinvestigated the effect of insulin on the accumulation of α-amino*iso*butyric acid by both intact and ordinary 'cut' diaphragm preparations, and have also studied the effect of insulin on the accumulation of a number of utilizable, naturally occurring amino acids.

METHODS

Radioactive materials. Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Glycine and α-amino*iso*butyric acid had ^{14}C in the carboxyl position; alanine, leucine, phenylalanine, arginine, lysine, aspartic acid and glutamic acid were all the L-isomers and uniformly (U) labelled with ^{14}C. The concentrations at which the amino acids were added to the medium are indicated in the tables and figures. In the experiments with intact diaphragm the amount of radioactivity added to the medium (irrespective of the quantity of amino acid) was 0·33 μc/ml.; in the experiments with cut diaphragm the amount of radioactivity added to the medium was 0·4 μc/ml.